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ON THE CHANGES AFFECTING
THE SOLUBILITY OF CASEIN

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ON THE CHANGES AFFECTING THE SOLUBILITY OF CASEIN

by

JOHN FREDERICK NICHOLSON

A Thesis Submitted for the Degree of

MASTER OF SCIENCE

UNIVERSITY OF WISCONSIN
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CONTENTS.

page.

Introduction .... - I.

History of Study of Cheese-Ripening................ 2.

Part I.

Object of the Present Study......................... 14.

Methods of Conducting the Experiment.............. 14.

Methods of Bacteriological Analysis............... 15.

Methods of Chemical Analysis...................... 16.

Total Soluble Nitrogen Determination.............. 16.

Amide Determination................ .... 17.

Effect of B. Acidi-lactici on Sterile milk........ 17.

Part II.

Effect of Low Temperature on the Digestive Processes in Milk................ 23.

1. Relation of Low Temperature to Bacterial Activities.................. 24.

2. Effect of Chloroform and Salt on the Digestive Processes in Milk......... 27.

    a. At Freezing Temperatures.................. 27.

    b. At the Higher Temperatures................ 29.

3. Relation of Low Temperature to the
Action of Rennet.................... 31.

General Summary............ .... 37.

Bibliography.................................... 38.

Changes Affecting the Solubility of Casein.

Introduction.

When a cheese undergoes the change, commonly spoken of as ripening, all of the ingredients are changed, in a measure, but the paracasein or green curd, is the constituent that is modified to the greatest extent. The casein in a freshly made cheese, or in a cheese that is known as a green cheese, is a tough indigestible mass. During the ripening process this curd becomes converted into bodies which are soluble in water and easily digested, and substances are produced which lend to the cheese the flavor and aroma so highly prized.

As to the cause of the production of this change in the casein there have been a variety of opinions advanced by different scientists.

From the time the modern study of bacteriology began, it has been generally assumed up to within a few years that the ripening of cheese is a process of bacterial growth; and the early experimenters hoped to determine the specific organism or organisms, capable of ripening cheese and use it as a pure culture starter in the same way the"Starter" is used today in the manufacture of butter. The more recent work has shown, however, that these changes are not produced wholly by bacterial growth, but are due in part, at least, to the action of certain unorganized ferments or enzyms.

The changes which occur in the proteid constituents of the cheese during its ripening are far more significant than any of the other changes. The whole value of cheese is dependent upon its ripening; a fact, that would make it extremely desirable that the exact knowledge of the manner of this ripening and its causes be understood.

History of the Study of Cheese-Ripening.

As early as 1875 Cohn(1) declared that the ripening of cheese was a fermentation due to the influence of fermenting organisms. After convincing himself that rennet as well as cheese contained numerous bacteria, he was of the opinion that the cheese ripening was due to those organisms that were introduced into the cheese by the addition of rennet. In the cheese he found such organisms as B. termo, micrococci and B. subtilis, the organisms that he believed were introduced with the rennet and were the agents in the subsequent ripening of cheese.

A few years later Duclaux(2) is convinced that the ripening is caused by the butyric ferment, because under its influence the casein of milk is precipitated and gradually dissolved.

Benecke(3) finds Cohn's rod-like bacteria in Emmenthaler cheese, and arrived at the conclusion that the ferment which brought about the peptonization of the fresh curd was B. subtilis, but that the formation of amides was not due to the action of the schizomycetes. In this connection it is also interesting to note that Benecke believed that the ripening might be due to the action of unorganized ferments, a fact, he said, which could be determined by making a cheese under the conditions that would exclude or prevent the activity

of living germs.

Duclaux(4) published his results in 1887 of further studies upon Cantal cheese, in which he claims to have isolated a large number of species to which he gave the name Tyrothrix, meaning cheese threads. All but one of these germs possessed the ability of coagulating the casein, and subsequently of digesting the curd. From one he even went so far as to isolate a ferment capable of rendering casein soluble. This enzym he called casease and the soluble product formed from the casein by its action, as caseone.

Adametz(5) examined in 1889 Emmenthaler and cottage cheese and isolated nineteen species, but among this large number he neither found B. subtilis, which Cohn had found, nor B. butyricus which Duclaux had found. However much the conflicting results baffled the endeavor to secure an organism capable of performing the work of cheese ripening, the bacteria were still considered the sole agents in the process.

The phenomenon of cheese ripening was classified by Adametz, and to each division of the operation he ascribed certain species of bacteria; for instance a certain class of organisms dissolves the paracasein while another group takes

the work up at this point and completes the decomposition of the proteids from the peptones and albumoses into amides and ammonia.

Benecke's suggestion that if bacteria did not play an important rôle in the cheese ripening they could be excluded, with no apparent effect, led Adametz to manufacture cheese, mixing with the curd such antiseptics as Thymol and Kreolin. As a result the curd did not ripen, demonstrating to him that beyond a doubt the bacteria were the cause of ripening, little suspecting that the strength of such antiseptics were also detrimental to the yet to be discovered unorganized ferments. It is well to recall here also, that Adametz worked with cottage cheese, the manner of manufacture of which excludes rennet and requires a considerable heating, a fact in itself sufficiently adequate, on the grounds of later knowledge, to explain the results obtained by him.

It was sufficient for the time, however, and cheese investigators for the next ten years were content to ascribe to bacteria the power of cheese ripening.

However much or little these early experimenters added to the knowledge of the cause of cheese ripening, the credit must be given to them for having aroused the attention of those investigators, who later were to figure so conspicuously in the effort to solve the problem.

Freudenreich(16) with the work previously done by Adametz, fresh upon his memory, followed step by step the ripening of a single cheese, in order to see if the changes the cheese passed through were the work of special bacteria, and to see if the species present at the commencement of ripening continued active until the end of the process. The cheese was analysed at intervals of eight to fifteen days, and like Adametz he found in fresh cheese many different micro-organisms which quickly disappeared as the cheese aged so that at the end of eighteen days, a microbe called Bacillus x, by the author, predominated; and at the end of 64 days, only this organism was found. The Bacillus x was a member of the lactic acid group. These results convinced Freudenreich that "the ripening of cheese was the work of bacteria; without bacteria there was no ripening". Later Adametz confirms these results by discovering that a germ very like the Bacillus x predominated in cheese.

As was stated it was supposed by Duclaux that the peptonizing bacteria were the chief cause of the digestion of casein which occurs in the ripening cheese. But it appears as shown by Freudenreichthat these peptonizing bacteria do not grow to any considerable extent in the cheese, the chief increase in bacteria being among the lactic acid organisms.

Freudenreich(7) in speaking of the Tyrothrix, potato or hay bacilli says "Far from multiplying in the cheese, they seem, even if added to it in great quantities, to die off rapidly, unless when added in the spore form, in which case they remain alive for a longer time, but without multiplying; and added to milk set for cheese, they seem neither to produce fermentation nor favor it.'"

That the bacteria, therefore, do multiply in the cheese is shown by many analyses. In the study of hard cheeses by Russell and Weinzirl(8) it appears that there are three periods of bacterial growth in the cheese ripening. At first there is a decline in the number of bacteria, continuing for about a day. This is followed by an increase in bacteria which continues for two or three weeks. This increase is due chiefly to the growth of the lactic acid bacteria, for while peptonizing bacteria are present they are in small quantities and this great increase appears to be wholly confined to the lactic acid group. After a time, the length of which is very variable the bacteria begin to decline, until finally only a comparatively small number are left.

When the peptonizing and gas producing bacteria are not eliminated the result is a product which, on account of its off-

flavor, or huffed apperance, is worthless as an article of food.

If bacteria aid in the chemical changes it would seem to be the lactic acid bacteria that are concerned.

Freudenreich(9) in 1891 said " it becomes, therefore our task, to isolate out of the cheese micro-organisms, by means of which, we could ripen cheese made from pasteurized milk, or which in sterile milk give off the same products which are to be found in cheese.

This interesting problem, apparently narrowed down to a single group of bacteria to be considered in the ripening of cheese, naturally was very attractive to the early investigators and we find a number of men attacking Freudenreich's lactic acid bacteria theory from all sides.

Schirokich(10) took up the study by preparing in milk pure cultures of peptonizing bacteria, and comparing the action of the two species on the casein of the milk. As a result he found that the casein was rendered almost entirely soluble by the former, while the latter did not in any way effect the casein, except to curdle it. These results naturally led him to remark that "even if the lactic acid germs are present in milk and cheese in any very great numbers, they do not induce the changes in the casein in the process of

ripening and if they exert any influence at all it is only indirectly, since these bacteria donot dissolve casein, do not give off ammonia and do not form the volatile acids characteristic of ripened cheese." The peptonizing bacteria on the other hand are of little value from the fact that Bonzinsky finds that there is in cheese only a small quantity of peptone which is not precipitated by ammonia sulfate while the peptonizing bacteria produce in the presence of casein almost pure peptones. The meager supply of these germs, also, in the cheese mass at any time during its ripening, does not indicate that they have much to do with the decomposition of the casein.

The question, Can the lactic acid bacteria contribute to the advancing digestion of the casein?, found negative and affirmative replies.

Freudenreich(11) remarks, in connection with the fact that the digesting organisms of Duclaux are found for a short time in cheese, "It may still be argued that during the short time which they lived in the substratum, so many of Duclaux's ferments of casein, called casease, were produced that by this means were possible the peptonization of the casein" "But", he says further, "if this were the case then would cheese, made from ordinary milk, and above all from

pasteurized milk, inoculated with large masses of liquifying bacteria, have ripened. This is found not to occur, surely then the lactic acid ferments play with the ripening the most important, if not even the whole part."

In 1897 (12) he seems to have thought to settle all disputes by growing lactic acid germs in sterile milk to which chalk had been added. The object of the chalk being to neutralize the lactic acid formed by the bacteria, giving the germ the opportunity for continued growth and multiplication. At the end of several months he found that the soluble nitrogen had actually increased several fold.

Practical experimenters had shown that cheese made from milk inoculated with lactic acid bacteria ripened normally, while such a result does not appear when it is inoculated with other types of bacteria. All of these facts lead to the conclusion generally accepted, that in cheese ripening as in cream ripening the lactic acid bacteria were chiefly concerned.

From 1897 to the present time a new factor has been considered in the cheese ripening process.

It had long been known that, enzyms could operate in the decomposition of proteid material, but it was not generally conceived that these unorganized ferments might be the sole cause of the cheese ripening, while the micro-organisms were incidental, and if

they played any part, did so by indirect methods , offering perhaps conditions favorable for the action of the enzyms.

Benecke almost a decade before this time had conceived the idea that the unorganized ferments played an important rôle, but further than this the question had not been attacked, only in so far that Adametz prepared cheese charged with strong antiseptics and found no ripening, a fact that had much to do with the exclusion of this factor for the next ten years, or until Babcock and Russell report the discovery of a natural ferment in milk direct from the cows udder, capable of rendering the casein soluble. In 1897 Babcock and Russell (13) named this enzym discovered by them, galactase, and claimed for it the power, in the presence of chloroform, conditions suppressing bacterial activity, to convert the insoluble nitrogenous compounds of milk into soluble albumoses and peptones. This discovery changed the conceptions relative to the question of cheese ripening.

They showed that cheese could ripen if kept in a perfectly sterile condition by the action of antiseptic agents, or by lowering the temperature to the degree that would exclude germ life.

It was found that galactase was closely allied to trypsin in its activity. It differs however in that it (galactase)

gives rise to a certain amount of free ammonia, and differs in regard to its optimum temperature of activity.

About this time Jensen(13a) discovered that cheese made from pasteurized milk to which ether and a certain amount of pancreas had been added cured quickly, and contained nearly 50% more soluble nitrogen than cheese made without the addition of pancreas.

Schirokich(14) in 1898 grew *Tyrothrix tenuis* for four days at 35° C and then filtered out the bacteria and added the germ-free filtrate to sterile milk. The casein was digested, but the milk did not obtain the cheese odor. He then grew a culture of pure lactic acid germs in sterile milk, and as soon as the milk had coagulated, added 5% of the sterile enzym from tyrothrix and incubated at 35°C. At the end of 15 days the mixture had a typical cheesy smell. Furthur experiments showed that the intensity of the cheesy odor depended upon the amount of acid present in the milk when the enzym was added. This fact suggested that enzym was the active principle and that lactic acid germs, to furnish acid, and digesting bacteria, to produce enzyms could be used for cheese ripening.

Babcock and Russell(15) in 1900 came to the conclusion that an increase in the amount of rennet extract used in mak-

ing cheese increases the amount of soluble nitrogenous products, which measure the progress of cheese ripening.

The galactase theory of Babcock and Russell was not to be accepted without opposition. Boekhout and de Vries(16) made cheese from milk obtained almost in a sterile condition from the cow, and failed to get ripening, concluding at once that the galactase theory of Babcock and Russell is incorrect.

Freudenreich(17) met the theory half way by saying, that the natural ferment perhaps participates in the ripening by rendering the casein soluble, and this facilitates the operation of the lactic acid ferments. And Jensen(17a) remarks in the same connection, as the result of his investigations that the chief function of galactase is in its rendering albuminoid substances soluble and the principal rôle of lactic ferments is to form decomposition products.

VanSlyke, Harding and Hart(18) concluded from a study of chloroformed cheese that" there is some agent at work in normal cheese which is not active in cheese made with chloroform.

This practically covers the history of the cheese ripening problem to the present time, and whether an application of bacteriology to cheese making will be possible cannot

yet be determined. The most recent work indicates, however, that the micro-organisms are needed to produce the proper flavors but the enzyms are also needed to induce the normal chemical changes which should take place during the ripening.

PART I.

Object of the Present Study.

The present work was undertaken for the purpose of determining the action of the lactic acid bacteria on the proteid compounds in milk to which calcium carbonate had been added. Since Freudenreich found that an organism of this class could actually convert insoluble nitrogen compound into the soluble forms in the course of a few months, its presence in cheese in such large numbers must not be without effect. The action of other than the pure lactic acid bacteria is not considered in this connection.

Methods of Conducting the Experiment.

The milk used for this experiment was obtained from the Experimental Farm herd, having been separated at the barn immediately after milking. The separated milk was taken immediately to the laboratory where flasks were filled each containing 400cc and placed in the autoclave for 15 minutes at a temperature of 120°C. In most cases the high heat slightly browned the sugar.

The calcium carbonate was freshly prepared from calcium chloride and sodium carbonate. These two salts were dissolved in water and sterilized separately in the autoclave, and

when they were cool mixed . The precipitated calcium carbonate was then washed with sterile water until the washings were completely neutral.

20 grams of the sterile calcium carbonate were added to 400cc of the milk, and the mixture allowed to incubate for two weeks to test the sterility.

Samples of milk with no calcium carbonate together with the carbonated milks, prepared in the above manner, were inoculated with a pure culture of Bacterium acidi-lactici, which had been grown in the laboratory for some time but just previous to inoculation had been passed through milk for the purpose of rejuvenescence. These flasks were incubated in a room kept at a constant temperature of 27°C.

Whenever samples were analysed all the milk in a flask of 400cc was used so as to deminish errors in sampling. This sample was analysed bacteriologically and chemically.

Methods of Bacteriological Analysis.

This side of the analysis required the plating of diluted samples, for the purpose of determining; first the purity of the culture, for any contamination necessitating the destruction of the sample; and second the number of germs per cubic centimeter.

The plates were always made with 12% gelatin, having an

acidity of 1.5% (Fuller Scale) and incubated at 20°C for four days.

Methods of Chemical Analysis.

The chemical analysis consisted in the determination of the degree of acidity and the amount of soluble nitrogen compounds.

The whole 400cc of the milk in the flasks were used in these determinations.

The acidity was determined by titration and the acid present converted into percentage of lactic acid.

For the soluble nitrogen determination 10cc of acetic acid (10%) are added to the flask of milk and boiled for two or three minutes. This process precipitates all of the insoluble proteid compounds leaving the soluble in solution. The mass is filtered through a paper filter and washed until the filtrate amounts to exactly 500cc. This solution then contains all of the soluble nitrogen represented in the 400cc of milk, so that 25cc of this filtrate will represent 20cc or grams of the original milk.

Total Soluble Nitrogen Determination.

25cc of the above filtrate is used to determine the nitrogen content according to the Kjeldahl method. The amount of nitrogen obtained represents the amount of soluble

nitrogen in 20 grams of the milk.

Amides- Determination.

50cc of the above filtrate is acidified with sulfuric acid and treated with phosphotungstic acid, and the whole mass is made up to 100cc. This is allowed to stand 30 minutes to insure complete precipitation of the nitrogen capable of being precipitated by phosphotungstic acid. After filtering, 50cc of the filtrate is used for the nitrogen determination according to the Kjeldahl method. The nitrogen obtained represents the amides present in 20 grams of the milk.

The figures given in all the tables represent the percentage of the nitrogen compounds in the milk as compared with the total amount of milk.

Effect of B. Acidi-lactici on Sterile Milk.

It has already been said that Freudenreich was able to grow lactic acid bacteria in sterile milk, in the presence of sterile chalk, and obtain an increase in the total soluble nitrogen, at the end of a few months.

The following is briefly the method used by him:

Milk to which chalk has been added was sterilized in the autoclave and inoculated with lactic acid germs derived from various sources. The chalk prevented the precipitation of the casein by neutralizing the lactic acid as it was pro-

duced by the organisms. It gave also adequate facilities for the continued growth and multiplication of the bacteria. At the end of several months these milks were passed through a Chamberland filter and the nitrogen determined in the filtrate as soluble nitrogen. The fact that he obtained as high as 0.22% of soluble nitrogen after the incubation, as compared to the initial 0.03%, convinced him that his germ theory was correct and that cheese ripened solely through the fact that the lactic acid germs were present.

The following tables show the results obtained by Freudenreich:

(19) "Freshly sterilized milk contained on an average 0.033% of total soluble nitrogen."

Organism used	Time in mos.	Total Sol. N.	Amides.
ε	9	0.235%	0.173%
ε	13	0.222%	0.151%
ε	13	0.246%	
α + δ	14	0.214%	0.140%
δ	9	0.094%	0.053%

Aside from the fact that cheese, composed in its green state of precipitated casein and fat, is far from being comparable to the milk in Freudenreich's experiment, it certainly did appear to the casual observer that bacteria

played a very important rôle. The lactic acid germ has never been credited with having the power, under any conditions, of secreting a proteolytic enzym. Any action it may have upon the casein being due entirely to its direct activity.

The following tables give the results of the experiment, and show the changes that have taken place in the milk at the ends of varying lengths of time.

The uncarbonated milks soon curdled and presented the appearance of a solid curd floating in a clear whey. While, on the other hand, the carbonated milks never curdled, but the millions of bacteria per cc, at the time of analysing in each case, testify to the fact that, these germs found conditions which were favorable to their growth. The calcium carbonate was not all used up, but it is to be noted in the tables that the milk was never neutral a condition that Freudenreich did not obtain. His milks were always neutral or slightly alkaline. The acidity of the carbonated milk was not due to free acid, but to acid salts which could not be neutralized by the free calcium carbonate. The organizm used was in all probability not identical with Freudenreich's organisms, but Bacterium acidi-lactici, being the common bacterium present in cheese during its increaseperiod, it ought to be able to act upon the insoluble casein here, if it plays the important rôle

ascribed to it in cheese ripening.

The figures represent the percentages of total soluble nitrogen and amides in the milk at the ends of the periods indicated.

Table I.

NO $CaCO_3$					With $CaCO_3$				
No Dys	T. sol N.	Amides	Bact. per cc	Acidity	No Dys	T. sol N.	Amides	Bact. per cc	Acidity
0	.073	------	------	.14%	0	.070	tr	------	--------
"	.070	------	------	"	"	.069	"	------	--------
52	.068	.006	---	.58%	52	.109	.011		.45%
	.056	.011	num.			.109	.011		
72	.051		12000	1.03%	338	.076	.024		
						.057	.025		
470	.07	.017		.72%					
	.059	.024							
470	.063	.016		.55%					
	.049	tr.							

Table II.

0	.044	.019		.14%	0	.048	.02		.14%
	.046	.018				.049	.02		
59	.066	.016	11mil.	.45%	28	.090	.02	96 mil	.49%
	.063	.015				.109	.014		
450	.051	.007		.45%	257	.063	.011	1 mil	.30%
	.042	tr				.061	tr		
					314	.066	.014		
						.060	.016		
					450	.063	tr		.17%
						.070	tr		

Table III.

					0	.054	.010		.14%
						.050	.016		
						.065	.012		
					72	.059	.029	640Mil	.22%
						.065			
					72	.071			

compounds quite as much as acid solutions. The fact that Freudenreich's milks were always neutral or alkaline would seem to partially explain the results obtained by him.

Three conclusions that can be drawn from the above tables are these:-

Ist. Calcium Carbonate prevents the accumulation of lactic acid giving the organisms facilities to continued growth and multiplication.

2nd. B. Acidi-lactici does not attack the precipitated casein of the uncarbonated milk.

3rd. B. Acidi-lactici does not convert the casein into soluble products when calcium carbonate is present.

Table IV.

No $CaCO_3$					With $CaCO_3$				
No Dys	T. sol N	Amides	Bact. per cc	Acidity	No Dys	T. sol N	Amides	Bact. per cc	Acidity
0	.038 .040	.015 .017			0	.050 .045	.016 .015		
325	.045 .052	.014 .012	200		325	.057 .059	.014 .014	6680000	
325	.082 .079	.028 .023	122300		325	.055 .056	.010	7866000	

In some cases the tables show a slight increase in soluble nitrogen, where the calcium carbonate has been added, in others there is no increase. In the uncarbonated milks thereis absolutely no increase in soluble nitrogen in any case. This latter fact is to be expected however, since Chodat and Hoffman-Bang (20) has demonstrated that the lactic acid germs cannot attack the precipitated casein. They demonstrated this by growing lactic acid bacteria in precipitated sterile casein from which all the sugar had been removed, and finding no increase in soluble nitrogen, whatever In the case of green cheese, the paracasein more closely resembles the conditions as produced by these investigators than the casein does in the liquid milk.

The tables also show the enormous bacterial content of the carbonated milks.

Strongly alkaline solutions dissolves the proteid

Part II.

Effect of Low Temperature on the Digestive Processes in Milk.

In the eighteenth Annual Report of the Wisconsin Agricultural Experiment Station, Babcock and Russell report the ripening of cheese at a temperature of $15^{o}-30^{o}F$. In the words of the authors we find this statement, "Cheese were placed at a temperature that was kept below freezing ($15^{o}-30^{o}F$) for most of the time. They were removed at the end of 14 and 17 months, respectively and examined chemically as well as for quality. These cheese showed an almost perfect texture and an extremely mild flavor with no trace of any bitter or undesirable taste."

The current opinion has been that bacteria are incapable of growth at temperatures below freezing. The results of Babcock and Russell on cheese ripening are therefore of interest as it would seem, if this is true, that the ripening changes were under the control of non-vital ferments.

In order, however, to test crucially by experimental analysis whether bacteria can flourish at these abnormally low temperatures, the following series of tests were made.

1. Relation of Low Temperature to Bacterial Activities.

The object in view when the following study was begun was to determine the ability of bacteria to grow when kept in a freezing mixture.

The experiments were performed in the following manner:

Skim milk separated at the Experimental Farm dairy barn was brought, immediately after separating, to the laboratory and divided into two parts. To one part 6% of sodium chloride was added while the other was left unsalted. These two samples were placed in a freezing mixture of ice and salt and left in a refrigerator, which was kept at a temperature of about 8°C. It was necessary to pack the milk with fresh freezing mixture twice a week. This treatment kept the milk continually in a partially congealed condition, but did not keep the temperature below 0°C, except for a few hours immediately after packing, at which time it would run as low as -12° to-15° C.

When the cheese ripened in the cold storage as already mentioned, it is presumed that the liquid contents did not congeal, but due to the dissolved salts were kept in the cheese mass, allowing the micro-organisms, if present, to find a suitable liquid medium.

The sodium chloride was added to the milk in order to produce such a salinity in the milk that the liquid would not be congealed when subjected to a temperature of a few degrees below 0^oC.

The milks both salted and normal always presented a partially congealed appearance but this frozen mass was of a soft consistancy and possessed a large percentage of liquid substance.

The purpose that the salt was intended to serve was defeated, but since the milks partook to a large degree the nature of the conditions probably met with in the cheese kept at this temperature, it is legitimate to draw conclusions from the data at hand. These milks were analysed bacteriologically and chemically at the end of varying lengths of time with the result as shown in the following table:

Table V.

Raw milk. (in freezing mixture)

Check.					6% NaCL.				
No Dys	T. sol N	Amides	acid	bact pecc	No Dys	T. Sol N	Amides	acid	bact. per cc.
0	.037 .040	.018 .018	.14%						
66	.088 .090	.030 .032	.21%	num	66	.056 .054	.014 .013	.14%	numerous
187	.159 .162	.018 .017	.40%	mil	187	.138 .132	.014 .014	.34%	thous.
					243	.105 .107	.016 .014	.30%	4500000

A study of the table will show the following:

Ist. The bacteria, in both milks, multiply at a freezing temperature.

2nd. In both cases there was an increase in total soluble nitrogen, but the amount decreases as we pass from normal to salted milks.

3rd. In no case did the amides increase.

The continued growth in the milks is in direct accordance with the results obtained by Higgins (21). Higgins found that a large number of organisms can grow at 0°C. It is also in accordance with the results obtained by the author in working with water organisms at a freezing temperature (22). Numerous species were found to be able to grow and multiply at 0°C when suspended in water. There are undoubtedly many species commonly found in milk which are able to grow and multiply under these apparently adverse conditions.

The acidity increased in both of the milks with the bacterial growth, which fact indicated that the organisms were acid producers, and probably belonged to the lactic acid group, although this point was not determined.

Since the increase of soluble nitrogen is largely composed of the higher products of decomposition, it would

seem to indicate thatneither the bacteria, their enzyms, nor the inherent enzyms in the milk are able to form amides at this temperature, but that the by-products formed were confined entirely to the albumoses and peptones.

A comparison of the following table obtained by Babcock and Russell(23) in working on cold storage cheese will show that amides are produced in cheese at the low temperature; a result somewhat different from those that may be deducted from the above tables.

Age in months	15° F.		
	Amount of rennet used	T. sol N.	Amides.
10	3 oz	.60	.23
14 1/2	3 oz	.62	.24
10	6 oz	.63	.22
14	6 oz	.87	.24
6	9 oz	.70	.12
10	9 oz	.74	.22
14	9 oz	.95	.24

2. Effect of Chloroform and Salt on the Digestive Processes in Milk.

a. At Freezing Temperatures.

In view of the fact that in the previous experiments, where raw and salted milks were used, the soluble nitrogen increased, it became desirable to determine the relation the bacteria held to this digestive process. Consequently the same conditions were provided and milks were prepared in the same manner as in the previous experiment, except that

$2\frac{1}{2}\%$ chloroform was added to each sample. The antiseptic destroyed the bacteria, as the table will show, leaving the enzym, galactase, free to operate under these conditions. The following table gives the result of both the chemical and bacteriological analyses:

Table VI.

Raw milk. (in freezing mixture)

$2\ 1/2\ \%\ CHCl_3$					6% NaCl and $2\ 1/2\%\ CHCl_3$.				
No dys	T. sol N	Amides	acid	bact per cc	No dys	T. sol N	Amides	acid	bact. per cc.
0	.037 .040	.018 .018	.14%						
66	.075 .071	.024 .019	.14%	0	66	.056 .064	.040 .028	.84%	0
187	.054 .046	.019 .019	.13%	12 10	187	.080 .084	.020 .021	.13%	0
243	.075 .068	.007 .014	.14%	0	243	.092 .100	.012 .012	.10%	0

The increase of soluble nitrogen is indeed very slight in both samples of milk and might easily be considered as insignificant. This may mean, that galactase can operate in the absence of chloroform but not appreciably in its presence, under the cold conditions, or that the bacteria are responsible for a large share of the increase of soluble nitrogen in the previous experiment. But whatever factor is operative it can produce at the cold temperature the higher products of decomposition, but not the lower ones.

b. At the Higher Temperatures.

It was necessary now after these results had been obtained, to determine the effects of chloroform and sodium chloride upon the action of galactase when placed at 25°C.

Milk from the same soutce as all the milks used up to this time, was treated with $2^{1/2}$% chloroform and varying quantities of common salt. One lot received 2% salt, anohher 4% and another 6%, while one lot was treated with only the chloroform for the purpose of controlling the other results. The following table gives the results of this experiment.

Table VII.

Raw Milk (at 25°C)

Reagents used	no dys.	T. sol. N.	Amides	acidity	no. of germs per cc.
0	0	.043	.013	.14%	not determined
		.047	.013		
$CHcl_3$ + 0	93	.294	.040		none
	93	.260	.040		"
	280	.319	.075		"
	280	.317	.073		"
$CHcl_3$ +2% Nacl	93	.230	.048		"
	93	.236	.050		"
$CHcl_3$ +4% Nacl	93	.143	.026		"
	93	.130	.024		"
	280	.175	.038		"
	280	.177	.040		"
$CHcl_3$ +6% Nacl	280	.187	.036		"
	280	.180	.034		"

From the table it is apparent that there is a large increase in soluble nitrogen in every case, but no bacterial

growth. The amides have increased, a condition not met with at the freezing temperature.

By comparing the two tables we find in the low temperature experiment (Table VI.) that the raw milk plus $2^{1/2}$% chloroform after 243 days contained .07% of soluble nitrogen and .01% of amides as against an initial .037% of soluble nitrogen and.01% amides. In the high temperature experiment (Table VII.) we find the raw milk plus $2^{1/2}$% chloroform after 280 days contains .319% of total soluble nitrogen and .075% of amides as against an initial .047% of total soluble nitrogen and .013% of amides.

Also comparing the raw milks plus $2^{1/2}$% chloroform and 6% salt in the freezing mixture and those incubated at 25° C we find a marked difference. The freezing temperature allows but slight increase in soluble nitrogen and no increase in amides, while the milks kept at 25°C shows that the total soluble nitrogen has increased nearly five fold while the amides has increased three fold.

It is also apparent that galactase preforms its functions in the presence of $2^{1/2}$% chloroform at 25°C, but is retarded by the addition of sodium chloride, the greater amount of salt used the less conversion of non-soluble nitrogen into the soluble forms.

To summarize the results as obtained from the table.

Ist. Salt inhibits the normal action of galactase.

2nd. Galactase apparently acts normally in the presence of $2^{1/2}$% chloform at the higher temperatures.

In view of these conclusions it appears that galactase is more largely responsible for the production of soluble nitrogen in the raw milks with no chloroform, at the low temperature, than the bacteria which have multiplied in these milks. In the salted milk the amount of soluble nitrogen was less than in the unsalted milks, at low temperatures. Since salt retards the activity of galactase as shown in the above tables, it would seem to indicate that we had to deal with the same enzym in the frozen milks. Therefore the choloform together with the low temperature conditions inhibits the activity of galactase.

III. Relation of Low Temperature to the Action of Rennet.

Rennet extract is a mixture of at least two enzyms, rennin and pepsin . Experiments were preformed for the purpose of determining the effect of a freezing temperature upon the normal activity of these enzyms.

This experiment was conducted in the following manner:

[illegible], Skim milk from the farm separator was divided, one

part of which was boiled for the purpose of destroying the inherent enzym, galactase, and the other was treated in a raw condition.

0.1% (1:1000) rennet extract was added to each sample (raw and boiled) after which they were divided again, one half of each being acidulated with .3% lactic acid. It was desired to imitate as nearly as possible the cheese conditions.

These four samples were kept in a freezing mixture, in the same manner as previously described, bringing the temperature down to 0^{o} C for most of the time.

Samples from these milks were analysed chemically and bacteriologically at the end of 96 and 196 days respectively.

The following tables give the results of this experiment.

Table VIII.

Raw milk. (in freezing mixture)

.1% rennet					.1% rennet .3% lac. acid.				
No Days	T. sol N	Amides	acid	bact per cc	No dys	T. sol N	Amides	acid	bact.
0	.040	.016	.144%	not determined	0	.040	.016	.44%	not determined
	.039	.018				.039	.018		
96	.27	.019	.75%	1422000	96	.255	.019	.7%	1170000
	.28	.020			196	.260	.017	.7%	
196	.36	.057	.8%	7252000	196	.282	.028	.7%	7500000
	.365	.047				.286	.026		
196	.319	.058	.8%	2418000	196	.276	.033	.45%	14102000
	.329	.056				.273	.036		
196	.280	.036	.8%		196	.279	.025	.7%	5222000
	.290	.036				.271	.028		

Table IX.

Boiled milk. (in freezing mixture)

No dys	T. Sol N	Ami-des	acid	bact. per cc	No dys	T. Sol N	Ami-des	acid	bact. per cc.
0	.040	.016	.144%	not determined	0	.040	.016	.44%	not determined.
	.039	.018				.039	.018		
96	.14	.021	.144%		96	.229	.03	.7%	
	.147	.017	.144%			.280	.029		
196	.213	.007	.7%	56323000	196	.257	.023	.6%	483480000
	.220	.007				.260	.019		
196	.225	.019	.35%	20951000	196	.253	.038	.45%	24940000
	.217	.021	.35%		196	.252	.028		
196					196	.279	.035	.8%	10400000
						.265	.040		

From these tables it is evident that bacteria find conditions favorable for multiplication even at a temperature of 0°C. It is also evident that the insoluble casein of the milk has been rendered soluble, but that the decomposition has been confined again to the higher products. In no case has there been an appreciable increase in the amido-

compounds. The acidity has progressed apace with the increase in total soluble nitrogen in nearly every case, indicating again that the species of bacteria present probably belong to the lactic acid group. In the case of the raw milk, enzyms from three sources may operate, namely: The inherent enzym, galactase, the enzyms from bacterial activity, and the enzyms introduced with the rennet. In the boiled milks it is to be noticed that the greater acidity accompanies the greater amount of soluble nitrogen, a condition to be expected also, since the pepsin in the rennet extract acts more energetically in an acid medium.

The data at hand indicate that bacteria not only develop and multiply at freezing temperatures but produce the products of their metabolic activities as demonstrated by the production of acid.

The organisms so often at fault for off flavors in cheese are undoubtedly unable to grow at this temperature, a fact that will harmonize Babcock and Russell's results, in seldom obtaining a bitter or tainted cheese when cured in the cold storage, and the results laid down in this paper. The tolerance to cold varies with the different species of bacteria as commonly found in milk.

The fact that bacteria always multiplied in milk at

this low temperature may account for some of the products of decomposition, but it is hardly to be expected that they would function to any great extent if they cannot be ascribed as effecting the ripening of cheese kept at higher temperatures. In no case from the data at hand can the bacteria be taken as the sole agents in the conversion of the casein into the soluble form, but they are undoubtedly responsible for the production of the requisite acidity for the normal action of pepsin in the rennet experiments.

The presence of enzyms, whether produced by an animal cell or a plant cell, will insure the decomposition of proteid matter, if of a proteolytic nature, but their activity is affected by external conditions. Heat and certain strong disinfectants will destroy their activity entirely. It is not strange therefore to conceive that a very low temperature will at least impair the activity of these unorganized ferments, and cause them to fall short of their normal activities. Galactase is at the lower temperatures, apparently unable to produce the lower products of decomposition and to be able only feebly to produce albumoses and peptones, but further experimental data is desired before this may be positively stated.

Conn (24) remarks: "The cheese flavors give this material its peculiar value as a food product. The origin of these flavors is therefore a matter of the greatest importance. Although the chemical changes which digest the casein in ripening cheese may be due largely to the chemical ferments, galactase and pepsin, the flavors have certainly a different origin. These enzyms do not produce flavors, and casein which has been rendered soluble by them does not have the characteristic cheese taste". In this we amy see the function of the micro-organisms, in that they not only furnish the requisite acidity for the action of certain enzyms, but lend to the cheese mass that peculiar taste and aroma, so characteristic of well cured cheese.

General Summary.

Bacterium acidi-lactici cannot render casein soluble either in the presence of calcium carbonate or in its absence. It does form acid which unites with the phosphates or the casein to produce acid salts, which in the cheese may give the requisite acidity for the activity of the rennet enzyms. Several species of the bacteria common to milk are able to grow, and produce their normal products, as shown by the production of acid, in milk kept in a partially congealed condition.

Galactase can operate in the presence of chloroform at 25°C, but cannot produce the lower products, as amides, and are enfeebled in the production of the higher products of decomposition.

The enzyms of rennet cannot produce the amido-compounds at 0°C, but the production of the higher compounds seem to be unimpaired.

Sodium chloride inhibits the action of galactase at 25°C in that a suppression of the production of all the products of decomposition is produced.

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